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New Design Strategy for Development of Specific Primer Sets for PCR-Based Detection of *Chlorophyceae* and *Bacillariophyceae* in Environmental Samples▽†

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Studying aquatic microalgae is essential for monitoring biodiversity and water quality. We designed new sets of 18S rRNA PCR primers for *Chlorophyceae* and *Bacillariophyceae* by using the ARB software and implementing a virtual PCR program. The results of specificity analysis showed that most of the targeted algal families were identified and nontargeted organisms, such as fungi or ciliates, were excluded. These newly developed PCR primer sets were also able to amplify microalgal rRNA genes from environmental samples with accurate specificity. These tools could be of great interest for studying freshwater microalgal ecology and for developing bioindicators of the health status of aquatic environments.

Microalgae play a key role in aquatic environments by forming the biomass base of the aquatic food web (1, 24). Monitoring them can provide accurate information about their biodiversity and natural succession with regard to biotic and abiotic factors (10, 16, 18, 22). Moreover, studying the ecology of these algae is a good way of evaluating the health of the ecosystem. For instance, diatomic indices can be used for estimating water quality (8, 23). Until now, the responses of freshwater communities to biotic and abiotic factors have been evaluated mainly by assessing algal biomass from pigment concentrations and from live-cell density (3). While the molecular approach constitutes a tool for quick and accurate monitoring of ecologically important organisms, most of the previously published probes or primers mainly targeted the eukaryotic domain, which does not allow conclusive phylogenetic affiliation or species identification. Previously published PCR primers designed at a deeper taxonomic level were only occasionally successful at discriminating targeted species from nontarget organisms (5, 21, 28, 29). With the addition of new gene sequences to the databases since 2005, it is now possible to design new PCR primers to a finer taxonomic level. Starting from published 18S ribosomal DNA (rDNA) sequences, our effort was focused on improving amplification PCR primers for the detection and monitoring of *Chlorophyceae* and *Bacillariophyceae*, two ecologically important groups of microalgae that are considered diverse and abundant in aquatic environments. A real-time PCR to assess the biomass of marine *Bacillariophyceae* was recently developed by designing a specific reverse primer for this class (7). However, in that study, PCR fragment length was especially chosen for quantitative development and, consequently, this tool was not appropriate for determining phylogenetic affiliation.

Our primer design was based on alignment of the 18S rDNA sequences with BioEdit software (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) using CLUSTAL W (9, 25). Two algal databases for *Chlorophyceae* (CHLORO) and *Bacillariophyceae* (BACI) were first generated, consisting of 132 and 94 18S rDNA sequences, respectively. They were then supplemented with a large number of sequences corresponding to other algal classes, as well as to ciliates, fungi, and plantae. The specificity of the primer sets designed was first tested in silico with the SILVA small subunit 16S/18S rDNA reference database released on 14 October 2008 and containing 279,862 complete or partial small subunit rDNA sequences listed in the ARB software package and database (http://www.arb-home.de) (14, 17) (see Table S1 in the supplemental material). Of 793 18S rDNA sequences belonging to the *Chlorophyceae* class, 65.8% matched with the primer ChloroF and 80.5% with the primer ChloroR, and only 13.3% did not match with either primer. All families were taken into account except the *Dunaliellaceae* family. Even though the specificity of CHLORO primers for the *Trebouxiophyceae* class was not complete, this primer pair targets several species of this class and, more specifically, the *Chlorella* species considered to be abundant microalgal species in freshwater ecosystems. Of 308 18S rDNA sequences of the *Bacillariophyceae* class, primer matches were 74.7% for BaciF and 96.4% for BaciR, and only 2.9% did not match either primer. Two families belonging to the *Coscinodiscophyceae* (*Biddulphiophycidae* and *Cymatosirophycidae*), another class of the diatom phylum, were also detected by the primer pair. In parallel, the probe check tool of the ARB package was used to evaluate the number of mismatches with the primer sequences (13) (see Table S2 in the supplemental material). According to the different analyses of specificity, the primers were considered useful molecular tools to detect main targets in the *Chlorophyceae* and *Bacillariophyceae* classes.

The specificity of the primer pairs was also assessed against

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TABLE 1. Total numbers of members of *Chlorophyceae* and *Bacillariophyceae* classes detected by using a virtual PCR program

Class	Subclass	Order[a]	No. of sequences	No. of targeted families/total no. of families in group
Chlorophyceae		*Chaetopeptidales*	2	1/1
		Chaetophorales	4	4/6
		Chlamydomonadales	220	9/14
		Chlorococcales	17	4/5
		Sphaeropleales	106	6/7
		Chlorosarcinales	12	3/5
		Tetrasporales	5	3/4
		Spermatozopsis	1	1/1
		Pseudomuriella	3	1/1
		Radiococcus	1	1/1
		Chlamydopodium	3	2/2
		Desmococcus	2	1/1
		Nautococcus	1	1/1
		Chlamydocapsa	1	1/1
		Dictyochloris	3	1/1
		Gloeocystis	1	1/1
Totals for *Chlorophyceae*			382	40/52
Bacillariophyceae	*Bacillariophycidae*	*Achnantales*	11	4/4
		Bacillariales	55	5/6
		Cymbellales	2	1/3
		Entomoneidaceae	3	1/1
		Naviculales	107	8/10
		Thalassiophysales	9	2/2
	Eunotiophycidae	*Eunotiales*	9	1/1
Totals for *Bacillariophyceae*			196	22/27

[a] Orders in class *Chlorophyceae* which are not targeted are *Oedogoniales*, *Cylindrocapsa*, *Elakatothrix*, *Microsporaceae*, *Mychonastes*, *Phaeophilaceae*, *Planktosphaeria*, *Pleurastrum*, *Pseudotetracystis*, *Treubariaceae*, and *Trochiscia*. Orders in class *Bacillariophyceae* which are not targeted are *Lyrellales*, *Surirellales*, *Rhopalodiales*, and *Asteroplanus*.

the NCBI nucleotide sequence database (12 September 2008) (ftp://ftp.ncbi.nih.gov/blast/db/FASTA) by using a virtual PCR program developed for this study. This program allows testing of the specificity of the primers considered to be pairs against the NCBI database. The program is written in Visual C++, and a zip archive containing the source code and a compiled version is available. Briefly, the program performs a scored search with the direct sense primer. If the number of mismatches is below the chosen threshold, then the search is continued using the reverse sense primer. If not, a second run is done, using the reverse complement of the pair of primers. For speed optimization, care was taken to incorporate a maximum amount of conditional tests within the minimum number of loop structures. Despite an unbuffered reading of the huge database file (circa 25 Gbytes), the program uses up to 65% of a dual core CPU usage. It also takes into account one or more possible mismatches on either primer in order to evaluate the robustness of the primer's specificity. When the two primers targeting the *Chlorophyceae* were tested in our virtual PCR program, 382 sequences from 40 different genera were identified using a BLAST search (Table 1) (2). When the two primers targeting the *Bacillariophyceae* class were tested, 196 sequences corresponding to 22 different genera were identified. Nonspecific amplifications counted for only 5.4% (*Streptophyta*, *Coccidia*, *Prasinophyceae*, and *Ulvophyceae*) for *Chlorophyceae* and 3.8% (*Dinophyceae* and uncultured stramenopiles) for *Bacillariophyceae*. When a base pair was randomly degenerated on one of the two primer sets, specificity was conserved. Finally, the specificity of the primers was investigated using standard PCR amplification performed on a variety of algal and nonalgal cultured strains (Table 2). DNA extraction was slightly modified from the method of Fawley and Fawley (6). Universal eukaryotic 18S rDNA primers were used to test whether the extracted DNA was amplifiable (12). PCR amplifications were performed using 0.2 mM of deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.5 μM of each primer, and 1.25 units of *Taq* DNA polymerase. For CHLORO amplification, the PCR program consisted of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, with an additional 10-min cycle at 72°C. For BACI amplification, a similar PCR program was used, except that the annealing temperature was 66°C. The characteristics of the PCR primers are listed in Table 3. When tested by conventional PCR, the primer sets were specific for their target groups, excluding all of the nontarget organisms (Table 2).

To check the specificity of the primer sets in situ, environmental samples originating from two different freshwater ecosystems (France), the eutrophic Lake Aydat and the mesoeutrophic reservoir Grangent (4, 19), underwent 18S rDNA PCR cloning and sequencing as previously described (11). At least 50 putative positive recombinant clones from each library were randomly picked from different plates. Then, PCR amplifica-

TABLE 2. Specificity of primer sets examined by conventional PCR amplification

Domain	Taxonomy or phylogeny[a,b]			Source[c]	Result for axenic culture[d]	Primer specificity (no. of mismatches) or PCR result with indicated primer set[e]					
						CHLORO			BACI		
	Class	Inferior taxonomic rank(s)	Species			F	R	Gel	F	R	Gel
Eukarya	*Chlorophyceae*	*Sphaeropleales*, *Selenastraceae*	*Ankistrodesmus fusiformis*	SAG	+	1	0	+	2	2	−
		Chlorococcales, *Hydrodictyaceae*	*Pediastrum tetras*	SAG	+	0	0	+	2	3	−
		Chlorococcales, *Hydrodictyaceae*	*Pediastrum duplex*	Lab	−	0	0	+	2	3	−
		Chlorococcales, *Hydrodictyaceae*	*Sorastrum* sp.	Lab	−	0[f]	0[f]	+	2[f]	3[f]	−
		Chlorococcales, *Scenedesmaceae*	*Scenedesmus ovalternus*	SAG	+	0[f]	0[f]	+	2[f]	4[f]	−
		Chlorococcales, *Scenedesmaceae*	*Coelastrum* sp.	Lab	−	0	0	+	10	10	−
		Chlorococcales, *Scenedesmaceae*	*Crucigenia apiculata*	SAG	+	NA	NA	+	NA	NA	−
		Chlorococcales, *Dictyosphaeriaceae*	*Dictyosphaerium pulchellum*	Lab	−	1	0	+	10	10	−
		Volvocales, *Volvocaceae*	*Volvox aureus*	Lab	−	0[f]	0[f]	+	2[f]	2[f]	−
		Chaetophorales, *Schizomeridaceae*	*Schizomeris leibleinii*	CCAP	−	0	0	+	2	3	−
	Trebouxiophyceae	*Chlorellales*, *Oocystaceae*	*Oocystis solitaria*	SAG	+	3	0	−	3	3	−
		Chlorellales, *Oocystaceae*	*Chlorella* sp.	Lab	−	1	0	+	3	3	−
	Bacillariophyta	*Bacillariophyceae*, *Bacillariophycidae*, *Thalassiophysales*	*Amphora coffeaformis*	CCAP	−	11	10	−	0	0	+
		Bacillariophyceae, *Bacillariophycidae*, *Cymbellales*	*Gomphonema parvulum*	SAG	+	10	9	−	0	1	+
		Bacillariophyceae, *Bacillariophycidae*, *Naviculales*,	*Phaeodactylum tricornutum*	SAG	+	11	10	−	0	0	+
		Fragilariophyceae, *Fragilariophycidae*, *Fragilariales*	*Fragilaria crotonensis*	Lab	−	12	10	−	0	2	+
		Coscinodiscophyceae, *Thalassiosirophycidae*	*Cyclotella cryptica*	CCAP	+	14	16	−	0	1	+
	Stramenopiles	*Synurophyceae*, *Ochromonadales*, *Ochromonadaceae*	*Ochromonas tuberculata*	CCAP	+	10	10	−	0	9	−
		Synurophyceae, *Synurales*, *Mallomonadaceae*	*Synura petersenii*	CCAP	+	10	10	−	0	9	−
	Cryptophyta	*Cryptomonadaceae*	*Cryptomonas* sp.	CCAP	+	8	10	−	9	10	−
	Alveolata	*Ciliophora*, *Intramacronucleata*, *Oligohymenophorea*	*Colpidium campylum*	Lab	−	10	10	−	8	10	−
		Ciliophora, *Intramacronucleata*, *Oligohymenophorea*	*Paramecium caudatum*	Lab	−	13	10	−	9	9	−
		Ciliophora, *Postciliodesmatophora*, *Heterotrichea*	*Blepharisma japonicum*	Lab	−	11	11	−	10	ND	−
	Euglenozoa	*Euglenida*	*Euglena* sp.	Lab	−	10	10	−	9	10	−
	Fungi/Metazoa group	*Basidiomycota*	*Rhodotolura* sp.	Lab	−	NA	NA	−	NA	NA	−
		Ascomycota	*Saccharomyces boulardii*	Lab	−	9	10	−	13	10	−
		Ascomycota	*Penicillium roqueforti*	Lab	−	15	14	−	19	12	−
		Ascomycota	*Aspergillus niger*	Lab	−	15	16	−	19	11	−
		Ascomycota	*Candida albicans*	Lab	−	15	ND	−	19	ND	−
		Fungi incertae sedis, *Mucorales*	*Rhizopus nigricans*	Lab	−	15	16	−	19	12	−
		Chytridiomycota	*Karlingiomyces* sp.	Lab	−	15	14	−	19	12	−
Bacteria	*Cyanophyceae*	*Chroococcales*	*Microcystis aeruginosa*	PCC	+	ND	ND	−	ND	ND	−
		Chroococcales	*Synecococcus* sp.	Lab	−	NA	NA	−	NA	NA	−
		Chroococcales	*Gomphosphaeria* sp.	Lab	−	NA	NA	−	NA	NA	−
		Oscillatoriales	*Planktothrix* sp.	PCC	+	ND	ND	−	ND	ND	−
	Gammaproteobacteria	*Enterobacteriales*, *Enterobacteriaceae*	*Escherichia coli*	Lab	−	ND	ND	−	ND	ND	−

[a] Members of the targeted classes (*Chlorophyceae* and *Bacillariophyceae*), as well as organisms outside of the defined target groups (*Trebouxiophyceae*, *Stramenopiles*, *Cryptophyta*, *Alveolata*, *Euglenozoa*, *Fungi/Metazoa*, *Cyanophyceae*, and *Gammaproteobacteria*), were taken as reference species for testing primer specificity.

[b] Taxonomic affiliations of organisms sensu stricto.

[c] Culture collections: CCAP, Culture Collection of Algae and Protozoa, Scotland, United Kingdom; SAG, Sammlung von Algenkulturen, Göttingen, Germany; PCC, Pasteur Culture Collection, Paris, France; Lab, laboratory culture collection.

[d] +, axenic culture; −, nonaxenic culture.

[e] (F, R) Primer specificity was tested in silico using the ARB software, and the number of mismatches to the 18S rDNA sequence of the species determined. The number of mismatches was determined for each primer. F, forward; R, reverse; NA, the sequence for the species or a related species within the same genus is not available; ND, the targeted region is not available in the sequence. (Gel) Binary result from conventional PCR, based on whether a band was detected by agarose gel electrophoresis. +, a band is clearly present; −, no band was observed.

[f] The number of mismatches corresponds to the results for another closely related species within the same genus if the sequence for the strain tested was not available in public databases.

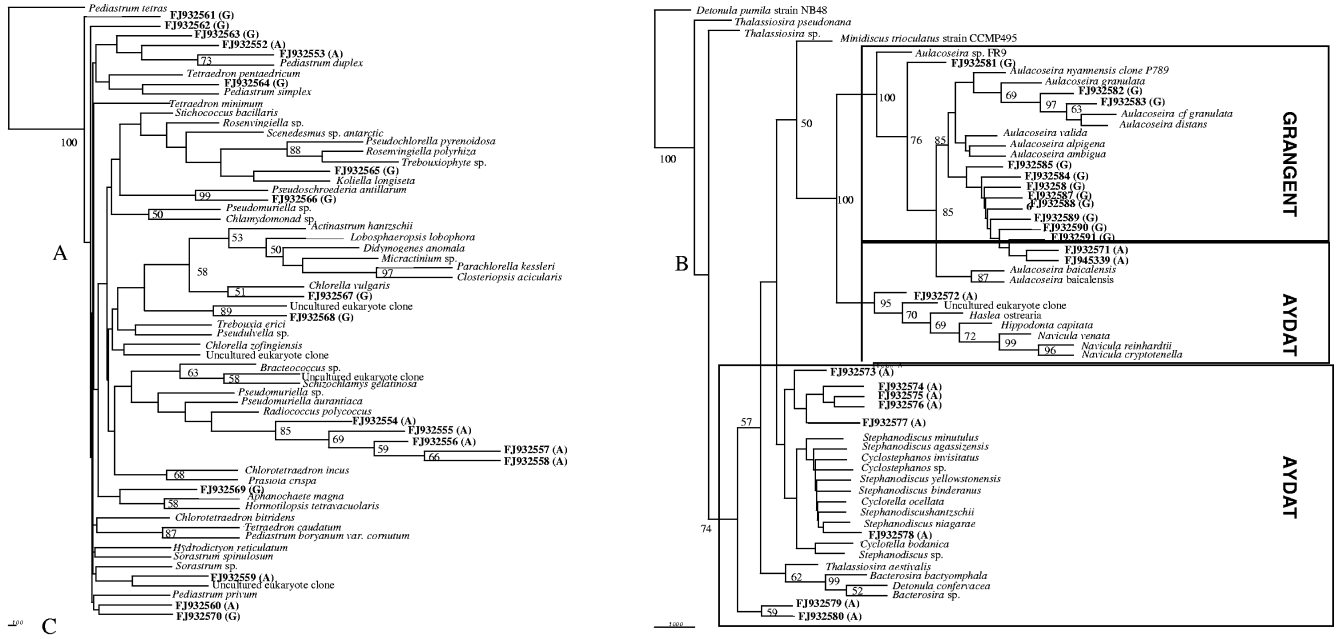
tions of the correct size were digested with the restriction enzyme MseI (Oxoid) for 12 h at 37°C and separated by electrophoresis in a 2.5% low-melting-point agarose gel (NuSieve). Two representatives of each clone producing the same operational taxonomic unit (OTU) were selected and sequenced from plasmid products. Finally, out of totals of 26 and 34 sequences originating from the CHLORO and BACI amplifications and submitted to a BLAST search, 19 and 22 gave rise to identification, respectively. An accurate specificity (≥96%) was obtained, and only organisms within the defined target groups were detected. The main OTUs originating from CHLORO amplification showed strong similari-

TABLE 3. Nucleotide sequences of the PCR primers designed and tested for amplification of the 18S rRNA gene from DNA of both algal cultures and freshwater environmental samples

Set	Primer	Sequence (5′→3′)	Length (bp)	G+C (%)	Theoretical T_m[a] (°C)	T_m[a] (°C) used in the study	Expected size of PCR product (bp)
CHLORO							
Forward	ChloroF	TGG CCT ATC TTG TTG GTC TGT	21	48	62	59	473
Reverse	ChloroR	GAA TCA ACC TGA CAA GGC AAC	21	48	62	59	
BACI							
Forward	BaciF	AGA TTG CCC AGG CCT CTC G	19	63	62	66	516
Reverse	BaciR	CCA TCG TAG TCT TAA CCA TAA AC	23	39	64	66	

[a] T_m, melting temperature.

ties to *Radiococcus polycoccus* (99%), *Pseudoschroederia reticulatum* (99%), and *Pediastrum duplex* (99%) for the Aydat lake library and to *Pediastrum* sp. (100%) and *Hydrodictyon* sp. (100%) for the Grangent reservoir library. Furthermore, OTUs linked to the *Bacillariophyceae* class demonstrated strong similarities to *Stephanodiscus minutulus* and *Cyclotella* sp. (100%) for the Aydat lake library and to *Aulacoseira* sp. (100%) for the Grangent reservoir library. The neighbor-joining (NJ) tree of



AYDAT		Relative abundance (RA) (%)	Cell/L	GRANGENT		Relative abundance (%)	Cell/L
Chlorophyceae	*Ankistrodesmus* sp.	1.1	129300	***Chlorophyceae***	*Ankistrodesmus* sp.	0.64	6000
	Tetraedron sp.	0.6	73897		*Scenedesmus* sp.	10.2	95000
	Kirchner sp.	0.2	18474		*Pediastrum* sp.	12	112000
	Elakatotrix sp.	0.3	36949	***Bacillariophyta***	*Aulacoseira granulata*	3.5	33000
	Staurastrum sp.	0.3	36949	***Cryptophyceae***	*Cryptomonas* sp.	17.8	166000
	Scenedesmus sp.	0.3	36949		*Rhodomonas* sp.	17.8	166000
Trebouxiophyceae	*Oocystis* sp.	2.3	277115	***Cyanophyceae***	*Anabaena* sp.	10.2	95000
Bacillariophyta	*Aulacoseira* sp.	0.8	92372		*Synechocystis* sp.	27.9	260000
	Cyclotella sp.	7.1	849827	**Total**		100	9332000 (percent error = 25.3)
	Fragilaria sp.	1.5	184745				
	Navicula sp.	0.3	36949				
Cryptophyceae	*Cyanomonas* sp.	1.5	184743				
	Rhodomonas sp.	3.3	387961				
Cyanophyceae	*Aphanothece* sp.	68.1	8128707				
	Synechococcus sp.	4.3	517281				
	Synechocystis sp.	2.3	277115				
	Oscillatoria sp.	3.9	461858				
	Microcystis sp.	0.6	73897				
Dinophyceae	*Trachelomonas* sp.	0.8	92372				
	Peridinium sp.	0.3	36949				
Total		100	11934419 (percent error = 7.71)				

FIG. 1. Analysis of environmental samples. (A and B) Phylogenetic analysis of 18S rRNA gene sequences showing the positions of clones obtained through PCR-based cloning from environmental samples from Aydat Lake and Grangent reservoir. The trees were constructed using the PHYLIP package and NJ method (20). Only bootstrap percentages (of 1,000 bootstraps) above 50 are shown. (A) Distance tree of sequences obtained from environmental DNA extracted and amplified with the CHLORO primer set designed in the study. The NJ unrooted trees were inferred from an alignment of 64 taxa with a character sampling of 445 nucleotides. (B) Distance tree of sequences obtained from environmental DNA extracted and amplified with the BACI primer set designed in the study. The NJ unrooted trees were inferred from an alignment of 59 taxa with a character sampling of 515 nucleotides. (C) Microscopic identification of microalgae (genus level). Ten-milliliter samples were sedimented, and algal density was evaluated by counting in 30 fields, using Utermöh's method (27). Percent error was calculated according to the method of Lund et al. (15).

sequences obtained from BACI amplification, but not that obtained from CHLORO amplification, revealed two main clusters according to the origin of the sample, which were strongly supported by their bootstrap values (Fig. 1A and B). As our sequence retrieval was in good agreement with observations of the microbial samples by epifluorescence microscopy, we conclude that our sequence retrieval was not significantly biased (Fig. 1C).

The development of these new primer sets will facilitate data acquisition from natural phytoplankton populations and give a better description of their biodiversity, which is notoriously difficult to study by simple microscopic observation. These primers will find their usefulness in different ways. They will allow the detection of new species and, consequently, the improvement of the robustness of phylogenetic reconstruction. Moreover, they could also be very useful for studying the dynamics of algal communities in the context of pollution, such as that caused by exposure to herbicides (26).

Nucleotide sequence accession numbers. The nucleotide sequences reported here have been deposited in the NCBI GenBank database under accession numbers FJ932552 to FJ932591 and FJ945339.

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